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CUTTING EDGE

Cutting Edge: Inhibition of Hepatitis B Virus Replication by Activated NK T Cells Does Not Require Inflammatory Cell Recruitment to the Liver[1]

Kazuhiro Kakimi,*† Thomas E. Lane,‡ Francis V. Chisari,* and Luca G. Guidotti*[2]

We have previously reported that intrahepatic NK T cells activated by α-galactosylceramide inhibit hepatitis B virus replication noncytopathically in the liver of transgenic mice. This effect is mediated by antiviral cytokines directly produced by activated NK T cells and/or by other cytokine-producing inflammatory cells that are recruited into the liver. In this study, we demonstrated that IFN-γ produced by activated NK T cells induced parenchymal and nonparenchymal cells of the liver to produce high levels of CXC chemokine ligands 9 and 10, which mediated the intrahepatic recruitment of lymphomononuclear inflammatory cells. Recruitment of these cells was not necessary for the antiviral activity, indicating that direct activation of the intrahepatic resident NK T cell is sufficient to control viral replication in this model. ***The Journal of Immunology,*** **2001, 167: 6701–6705.**

We have previously reported that hepatitis B virus (HBV)[3] replication is inhibited noncytopathically in the liver of HBV-transgenic mice by NK T cell activation following a single injection of α-galactosylceramide (α-GalCer) (1), a glycolipid Ag presented to $V\alpha14^+NK1.1^+$ T cells by the nonclassical MHC class I-like molecule CD1d (2). Within 24 h of α-GalCer injection, antiviral cytokines such as IFN-γ are detected in the liver of these animals and HBV replication is abolished (1). This is associated with the recruitment of activated NK cells and T cells into the organ and the rapid disappearance of NK T cells from the liver, presumably reflecting their activation (1). Based on these results, it is possible that α-GalCer inhibits HBV replication by directly activating NK T cells to produce antiviral cytokines. Alternatively, antiviral cytokines could be produced in the liver by recruited inflammatory cells.

The recruitment of inflammatory cells into the liver following injection of α-GalCer is likely to be regulated by NK T cell-derived cytokines (i.e., IFN-γ) that induce the intrahepatic production of chemokines. At this time, the chemokines that are induced in the liver following NK T cell activation have not been defined. CXC chemokine ligand (CXCL)9 (monokine induced by IFN-γ) and CXCL10 (chemokine responsive to γ-2/IFN-γ inducible protein) are known to be induced by IFN-γ (3, 4), to bind the same chemokine receptor (CXCR3) (5), and chemoattract lymphocytes and monocyte/macrophages (3, 4). Both chemokines are produced by macrophages and recent studies have also shown that these same chemokines can be produced by primary hepatocyte cultures (6) or by hepatocytes in vivo (27). CXCL9 and CXCL10 are induced in the mouse liver during vaccinia virus infection (7) and they accelerate viral clearance and the associated hepatitis (8). CXCL9 also contributes to protection against hepatic mouse cytomegalovirus infection (9).

Collectively, the foregoing results suggest that CXCL9 and CXCL10 might play a role in the recruitment of inflammatory cells into the liver following NK T cell activation by α-GalCer. It is also possible that these chemokines could mediate the antiviral effects of activated NK T cells. To test these hypotheses, we performed a series of experiments aimed at monitoring the following: 1) the ability of α-GalCer to induce CXCL9 and CXCL10 in the liver; 2) the source, kinetics, and regulation of CXCL9 and CXCL10 expression; 3) the ability of α-GalCer to recruit inflammatory cells into the liver; and 4) the ability of chemokine-specific neutralizing Abs to block the proinflammatory and antiviral effects of activated NK T cells.

Materials and Methods

Mice

HBV-transgenic mouse lineage 1.3.32 used in this study have been previously described (10). In all experiments, the mice were matched for age (8 wk), sex, and hepatitis B e Ag (HBeAg) levels in their serum before experimental manipulations.

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[3] Abbreviations used in this paper: HBV, hepatitis B virus; α-GalCer, α-galactosylceramide; HBeAg, hepatitis B e Ag; NRS, preimmune normal rabbit serum; RPA, RNase protection assay; IHL, intrahepatic leukocyte; αIFN-γ, hamster neutralizing Abs specific for IFN-γ; CXCL, CXC chemokine ligand.

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Reagents

α-GalCer was kindly provided by Dr. Y. Koezuka (Kirin Brewery, Gumma, Japan) and prepared as previously described (1). Hamster mAbs H22 specific for murine IFN-γ was generously provided by Dr. R. Schreiber (Washington University, St. Louis, MO) (11). The control hamster IgG was purchased from Jackson ImmunoResearch Laboratories(West Grove, PA). Rabbit polyclonal Abs to mouse CXCL9 or CXCL10 and preimmune normal rabbit serum (NRS) have been previously described (12, 13).

Tissue DNA and RNA analysis

Total DNA and RNA were isolated from frozen livers and analyzed for HBV DNA by Southern blot, and for chemokine, chemokine receptor, cytokine, T cell and macrophage marker RNAs by RNase protection assay (RPA) exactly as previously described (10, 14, 15). The relative abundance of specific DNA and RNA molecules was determined by phosphor-imaging analysis, using Optiquant image analysis software (Packard, Meriden, CT).

In situ hybridization

This procedure was conducted exactly as described (10). For CXCL10, a 726 bp cDNA fragment was synthesized and cloned in pGEM4 (Promega, Madison, WI) as described previously (14). For CXCL9, a 302-bp cDNA fragment spanning nucleotides 101–402 of the murine CXCL9 gene (GenBank accession no. M34815) was synthesized by RT-PCR and after sequence verification cloned in pGEM4. The ^{33}P-labeled RNA probes used for in situ hybridization were generated by T7-driven transcription of 1 μg of linearized plasmids containing CXCL9- or CXCL10-specific sequences.

Histological analysis

For histological analysis, liver was fixed in 10% zinc-buffered formalin (Anatech, Battle Creek, MI), embedded in paraffin, sectioned (3 μm), and stained with H&E as described (1).

Isolation and analysis of intrahepatic leukocytes (IHLs)

IHLs were isolated from two liver lobes and analyzed by flow cytometry. Single-cell suspensions were prepared and analysis of the IHL population was performed by flow cytometry, exactly as described (1). The cells were surface stained with FITC- or PE-labeled anti-CD3, CD4, CD8, DX5, NK1.1, CD19, Gr-1, CD11b, or CD11c Abs (BD PharMingen, San Diego, CA). Samples were acquired on a FACSCalibur flow cytometer, and the data were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA).

Results and Discussion

CXCL9, CXCL10, and CXCR3 are induced in the liver following α-GalCer injection

We have previously reported that hepatic NK T cell activation by α-GalCer inhibits HBV replication in the liver of transgenic mice (1). This occurs within 24 h and is associated with the intrahepatic induction of antiviral cytokines such as IFN-γ (1). To determine whether the IFN-γ inducible chemokines CXCL9 and CXCL10 along with their common receptor CXCR3 were induced in the liver of transgenic mice injected with α-GalCer, we monitored the expression of CXCL9, CXCL10, and CXCR3 by RPA. HBV-transgenic mice (three mice per group) were i.v. injected with 10 ng of α-GalCer and sacrificed at the indicated time points (Fig. 1). Total hepatic RNA was extracted and analyzed and the results were compared with those observed in livers from transgenic littermates injected with saline (NaCl) that were sacrificed on day 1 after NaCl injection (Fig. 1).

As shown in Fig. 1 for two representative mice per group, the intrahepatic RNAs for CXCL9 and CXCL10 were strongly induced over 1000- and 200-fold, respectively, when compared by phosphor imaging analysis with preinjected levels by day 1 after α-GalCer injection. CXCL9 and CXCL10 transcripts decreased

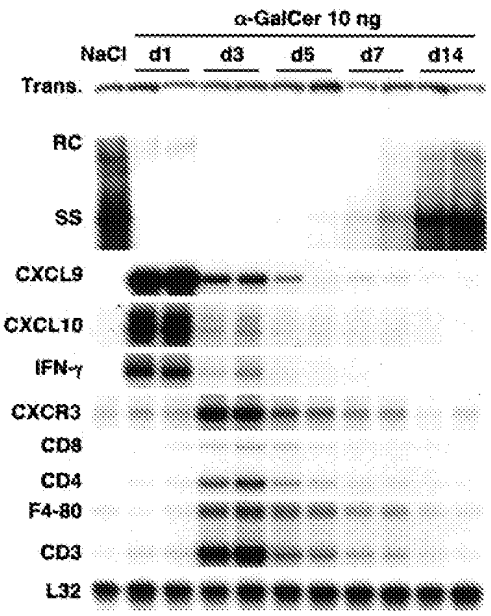

FIGURE 1. Intrahepatic expression of CXCL9, CXCL10, and CXCR3 after α-GalCer injection. HBV-transgenic mice (3 mice per group) were injected i.v. with 10 ng of α-GalCer and sacrificed at the indicated time points. Total hepatic DNA was analyzed for HBV replication by Southern blot analysis. Bands corresponding to integrated transgene (Trans.), relaxed-circular (RC), and single-stranded (SS) linear HBV DNA replicative forms are indicated. Total hepatic RNA was analyzed for the expression of chemokine (CXCL9 and CXCL10), cytokine (IFN-γ), chemokine receptor (CXCR3), and T cell-specific (CD8, CD4, and CD3) and macrophage-specific (F4-80) transcripts by RPA. The RNA encoding the ribosomal protein L32 was used to normalize the amount of RNA loaded in each lane. The results were compared with those observed in livers pooled from 10 age-, sex-, and serum HBeAg-matched transgenic littermates injected with saline (NaCl). Note that the indicated bands for CXCL9 and CXCL10 correspond to an exposure time of the autorad of 2 and 16 h, respectively.

thereafter returning to baseline levels by days 14 and 5, respectively (Fig. 1). The kinetics of CXCL9 and CXCL10 induction coincided with the induction of IFN-γ and the inhibition of HBV replication (Fig. 1) as also previously reported (1). CXCR3 RNA increased in these livers as well, albeit with delayed kinetics, reaching its peak by day 3 and returning toward baseline levels by day 7–14. This is consistent with our previous observation that by day 3 after α-GalCer injection, lymphomononuclear cells are maximally recruited into the liver and small, widely scattered necroinflammatory foci are detectable in the organ (1). In keeping with this, the signal for T cell (CD8, CD4, and CD3) and macrophage (F4-80) RNAs were maximal by day 3 (Fig. 1). Altogether, these results demonstrate that the IFN-γ inducible chemokines CXCL9 and CXCL10 are induced in the liver after NK T cell activation due to α-GalCer injection and they also suggest that these chemokines may be involved in the recruitment of CXCR3$^+$ inflammatory cells into the liver.

To determine which cells produce CXCL9 and CXCL10, the hepatic content of both chemokine RNAs was analyzed by in situ hybridization analysis of livers derived from HBV-transgenic animals sacrificed at day 1 after α-GalCer injection. CXCL9 and CXCL10 were detected not only in nonparenchymal cells (Kupffer cells and infiltrating inflammatory cells) but, even more abundantly, in parenchymal (hepatocytes) of the liver (not shown). This indicates that the hepatocytes represent a major source of CXCL9

and CXCL10 RNAs in the α-GalCer-injected livers and it is consistent with previous experiments that showed expression of CXCL9 and CXCL10 by the hepatocyte following the transfer of HBV-CTLs into HBV-transgenic mice (27) and by primary hepatocyte cultures derived from mice treated with IL-2/IL-12 (6).

IFN-γ mediates the antiviral activity of α-GalCer and the induction of CXCL9 and CXCL10 in the liver

To define the role of IFN-γ in the α-GalCer-dependent antiviral activity and in the intrahepatic induction of CXCL9 and CXCL10, we monitored the ability of hamster neutralizing Abs specific for IFN-γ (αIFN-γ) to modulate these processes. Two groups (three mice per group) of HBV-transgenic mice were injected once i.p. with 250 μg of either αIFN-γ or control hamster Ig 16 h before α-GalCer. Mice were sacrificed 48 h later and their livers were harvested for HBV DNA analysis by Southern blot and IFN-γ, CXCL9, and CXCL10 RNA analysis by RPA. The results were compared with those observed in livers pooled from 10 age-, sex-, and serum HBeAg-matched transgenic mice littermates injected with saline (NaCl).

As expected, HBV replication was suppressed in the mice that received α-GalCer and control Ig and this coincided with the induction of IFN-γ, CXCL9, and CXCL10 RNAs in the liver (Fig. 2, *left*). In contrast, HBV replication was still strongly detected in the livers of transgenic mice that were treated with α-GalCer and αIFN-γ and the induction of CXCL9 and CXCL10 was almost completely blocked (Fig. 2, *left*). These results confirm previous experiments that showed that IFN-γ mediates most of the antiviral activity of α-GalCer (1) and they also indicate that IFN-γ mediates the α-GalCer-dependent induction of CXCL9 and CXCL10 in the liver. The fact that the intrahepatic levels of HBV replication in αIFN-γ-treated mice were slightly lower than those observed in saline-injected controls (Fig. 2, *left*) suggests the existence of a marginal antiviral activity of α-GalCer which is independent of IFN-γ. This is consistent with the notion that IFN-αβ can also be induced by α-GalCer (1) and trigger IFN-γ-independent antiviral pathways in this model (15, 16).

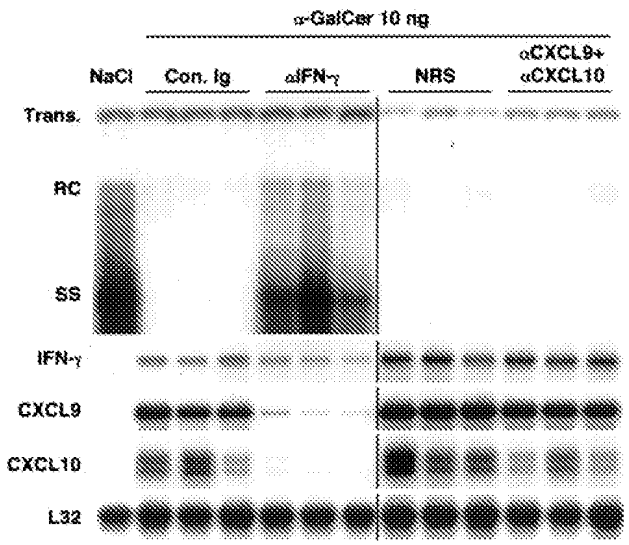

FIGURE 2. *Left,* IFN-γ mediates the antiviral activity of α-GalCer and the induction of CXCL9 and CXCL10 in the liver. HBV-transgenic mice were i.p. injected with 250 μg of hamster mAbs to IFN-γ and sacrificed 48 h after α-GalCer injection. Control mice were injected with 250 μg of irrelevant hamster IgG before α-GalCer injection and sacrificed at the same time. Total hepatic DNA was analyzed for HBV replication by Southern blot analysis and total hepatic RNA was also analyzed by RPA for the expression of IFN-γ, CXCL9 and CXCL10 as described in Fig. 1. *Right,* CXCL9 and CXCL10 do not mediate the antiviral activity of α-GalCer. One milliliter of either a mixture of anti-CXCL9 and anti-CXCL10 neutralizing rabbit Ig or NRS was administered i.p. into HBV-transgenic mice twice, first 16 h before and then simultaneously with 10 ng of α-GalCer injection. Forty-eight hours later, mice were sacrificed and livers were processed exactly as described above.

CXCL9 and CXCL10 do not mediate the antiviral activity of α-GalCer

To define the role of CXCL9 and CXCL10 in the α-GalCer-induced antiviral activity, we monitored the ability of rabbit neutralizing Abs specific for CXCL9 and CXCL10 to modulate this process. Two groups (three mice per group) of HBV-transgenic mice were injected i.p. with 1 ml of either a mixture of CXCL9 and CXCL10 neutralizing rabbit Ig (αCXCL9 and αCXCL10) or NRS twice, first 16 h before and then simultaneously with α-GalCer injection (10 ng/mouse). Mice were sacrificed and livers were harvested 48 h after α-GalCer injection and total hepatic DNA and RNA were analyzed as described above.

HBV replication was abolished in mice that received α-GalCer plus NRS as well as in mice that received α-GalCer plus Abs specific for CXCL9 and CXCL10, and this coincided with similar inductions of IFN-γ, CXCL9, and CXCL10 RNAs in the liver in both groups of animals (Fig. 2, *right*). These results indicate that CXCL9 and CXCL10 do not directly inhibit HBV replication themselves nor do they mediate the antiviral potential of α-GalCer in our system.

IFN-γ, CXCL9, and CXCL10 contribute to the recruitment of inflammatory cells into the liver

To determine the characteristics of intrahepatic inflammatory infiltrate in the same livers described in Fig. 2, the absolute number of IHLs recovered was quantitated and the phenotype of the recruited inflammatory cell subsets was determined by FACS analysis.

When compared with NaCl-injected controls, the total number of IHLs significantly increased in the liver of α-GalCer-treated mice that received control Abs (Fig. 3*A*). The total number of IHLs increased 7- to 8-fold at 48 h after α-GalCer injection. αIFN-γ or anti-chemokine treatments reduced the number of total IHLs recruited by ~2-fold (Fig. 3*A*). The most striking decrease was observed for $NK1.1^+CD3^-$ cells (Fig. 3*B*), which increased ~20-fold in control mice and αIFN-γ or anti-chemokine treatments reduced their number by ~5-fold (Fig. 3*B*). $CD8^+$ cells (mostly CTLs; Fig. 3*C*) and $CD4^+$ cells (mostly Th cells) increased ~4- to 7- and 3-fold in control mice, and αIFN-γ or anti-chemokine treatments reduced their numbers by ~2-fold (Fig. 3, *C* and *D*). $CD11b^+/CD11c^+$ cells (mostly myeloid dendritic cells) and $CD11b^+/CD11c^-$ cells (mostly macrophages) were also induced in control animals (~30- and 7-fold, respectively; Fig. 3, *E* and *F*) and αIFN-γ or anti-chemokine treatments reduced their number by ~3- and 2-fold, respectively (Fig. 3, *E* and *F*).

These results indicate that IFN-γ induced by NK T cell activation recruits different subsets of lymphomononuclear inflammatory cells into the liver and that most of this activity is mediated by the hepatic induction of CXCL9 and CXCL10. The recruitment of cell subsets ($NK1.1^+CD3^-$ cells, $CD8^+$ cells, and $CD11b^+/CD11c^+$ cells) that are known to express CXCR3 (3, 17, 18) was inhibited by both αIFN-γ and anti-chemokine treatments. The recruitment of $CD4^+$ T cells was also reduced by αIFN-γ but to a much lesser extent by the anti-chemokine treatment. Because $CD4^+$ T cells are known to express CXCR3

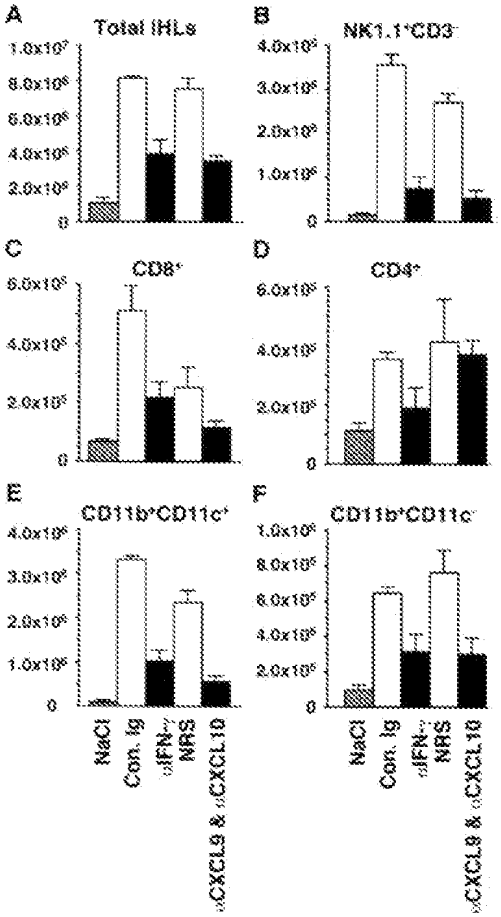

FIGURE 3. IFN-γ, CXCL9, and CXCL10 contribute to the recruitment of inflammatory cells into the liver. IHL analysis in the same animals described in the legend to Fig. 3. IHLs were isolated from two liver lobes of a known weight and analyzed by flow cytometry. The indicated number of total IHLs (*A*), NK1.1$^+$CD3$^-$ cells (*B*), CD8$^+$ cells (*C*), CD4$^+$ cells (*D*), CD11b$^+$CD11c$^+$ cells (*E*) and CD11b$^+$CD11c$^-$ cells (*F*) represent the numbers detected in whole liver.

(19, 20), these results may suggest that other chemoattractant factors inducible by IFN-γ play a more important role in the recruitment of these cell subsets into the liver. Similar reasons may explain the partially reduced recruitment of cells such as macrophages, which are not known to express CXCR3 (Fig. 3*F*). Despite a much higher number of recruited inflammatory cells, the intrahepatic levels of IFN-γ RNA in control mice were similar to those observed in mice treated with anti-chemokine Abs (Fig. 2, *right*). This indicates that the recruited inflammatory cells do not contribute to the intrahepatic production of IFN-γ and, thus, it is likely that most of the IFN-γ produced in the liver at this time point is the result of the direct activation of NK T cells by α-GalCer. Because IFN-γ mediates the α-GalCer-dependent inhibition of HBV replication (Fig. 2), these results suggest that the recruitment of inflammatory cells is not necessary to control HBV replication after NK T cell activation.

In conclusion, we showed in this study that IFN-γ secreted by α-GalCer-activated NK T cells rapidly and strongly induces nonparenchymal and parenchymal cells of the liver to produce CXCL9 and CXCL10. These chemokines recruit different cell subsets into the organ, particularly those that express CXCR3. Nonetheless, neither these chemokines nor the cells that they recruit mediate the IFN-γ-dependent antiviral activity of NK T cells, suggesting that the NK T cells themselves inhibit HBV replication in this system by secreting IFN-γ. It is known that activated NK T cells can recruit and activate NK in an-IFN-γ-dependent manner (21–23), raising the possibility that NK cells are actually responsible for most of the antiviral activity of NK T cells (24). Our results suggest that NK T cells themselves can play a direct antiviral role if they are sufficiently activated. Because it is apparent that the percentage of resident intrahepatic NK T cells is much higher in mice (~30%) (25) than humans (~4%) (26), the extent to which naturally or artificially activated NK T cells could contribute to the control of viral replication in humans remains to be determined.

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